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ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

Sixteenth Quarterly Report of Progress

Research Project R-36-015-001

January 1, 1969 - March 31, 1969

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Introduction

In several aspects of our recent work, we have been unable to obtain reproducible destruction rate curves for Bacillus subtilis var. niger and this has stopped significant progress in our studies on the characterization of the nature of the destruction rate curve with survivor numbers between approximately 10^{3} and 10^{-2} organisms per sample. Our work as well as extensive work by others has shown that moisture has a profound effect on spore heat resistance and we felt that studies centered around identifying basic information on moisture would be useful in identifying the source or sources of our problems as well as make a significant addition to our knowledge of the parameters governing water uptake and loss from spores.

To this end we concentrated our efforts this quarter on studies of (a) determining spore moisture content during various phases of our spore preparation process, (b) determination of moisture content of the can-matrix system used during heat resistance determinations, (c) effect of moisture in the environment on spore water content, (d) influence of moisture on the thermal destruction of Bacillus subtilis var. niger and (e) effect of spore lyophilizates on the z_D of Bacillus subtilis var. niger.

I. DETERMINATION OF SPORE MOISTURE CONTENT DURING DRYING AND EQUILIBRATION

In the process used in this laboratory for preparation of spores for inactivation by dry heat, the spores are first air dried for 24 hrs. at 40°C and this is followed by drying with flowing dry nitrogen for 24 hrs. at 40°C. After the drying stages, the spores are placed in an equilibration cabinet set to maintain a 41% R.H. at 90°F. and they are equilibrated for 24 hrs. before being used in heat inactivation determination. Approximately 15 mg of wet spores were placed in a small tinplated cup and were assayed for moisture content after each of the stages of spore preparation (Table 1). The data showed that 24 hrs. treatment with flowing nitrogen at 40°C was sufficient to assume dryside equilibration of spores and that a second period of 24 hrs. of equilibration (41% R.H. -90°F) had little effect on spore moisture content. From these data we have fixed the minimum drying and equilibration times at 24 hrs. each.

All moisture determinations done in this and subsequent sections of this report were made with a commercial moisture analyzer described in our last quarterly report.

II. DETERMINATION OF MOISTURE IN THE CAN-MATRIX SYSTEM USED FOR HEAT INACTIVATION DETERMINATIONS

Since metal surfaces will adsorb water, we measured the moisture content of the can-matrix system which we developed for holding tubes of dry spores during heat inactivation in an oil bath. Cans and matrices were dried and equilibrated by the same procedures used for spores.

After the equilibration step, the cans were sealed, fittings were installed so that dry N2 could be passed through the can, and the total moisture was determined that would be available upon heating to 125°C. Initial measurements indicated an excess of water in the cans with matrices above that calculated for air equilibrated at 41% R.H. at 90°F. A series of moisture determinations were made which showed that the matrix gave up relatively large amounts of water during heating and that this amount of water depended on the condition of the aluminum surface. Anodizing the aluminum increased its water adsorbing capacity and covering with epoxy did not prevent the adsorption of significant amounts of water. Since a matrix could not be used, a stainless steel spring clip was made to hold the sample tubes around the inside periphery of the can. This clip did not add significant amounts of water to the system and was adopted for use in routine thermal inactivation determinations. These data are summarized in Table 2.

III. EFFECT OF MOISTURE IN THE ENVIRONMENT ON SPORE WATER CONTENT

The procedure used for these experiments, unless otherwise indicated, was as follows: About 2 mg of (± 0.05 mg) of air dried spores was weighed into a small metal cup sized to conveniently fit in the moisture analyzer oven. The sample was dried in the analyzer at 105°C until the rate of moisture loss was less than 1 μg of H_2O/min. At this point the total moisture content of the spores was less than 1 μg of H_2O/mg and was considered "dry" and ready for sorption studies.

The viability of spores was not adversely affected by this procedure. Three small humidity chambers were constructed to provide relative humidities of 3%, 43% and 100% at 25°-27°C. They were designed to allow exposure and removal of the spore samples without greatly upsetting the equilibrium of the system, and at the same time to facilitate moving of the sample from the humidity chamber to the drying oven of the moisture analyzer in a minimal period of time. Although the system was reasonable satisfactory, it was limited to a relatively narrow range of temperatures and humidities. New equipment is being designed to overcome some of these limitations and will be described in a later report. The dried spore preparations were placed in the various relative humidity cabinets for different lengths of time, and then quickly moved to the moisture analyzer where the amount of water sorbed was determined by measuring the total amount of water desorbed from the sample at 105°C.

The results of the studies on the uptake and loss of water vapor by Bacillus subtilis var. niger are summarized in Figures 1, 2 and 3. From Figure 1 it is seen that the rate of uptake was rapid and that equilibrium conditions were reached in less than 30 minutes. The equilibrium concentration of water in the spores was proportional to the R.H. at which they were exposed.

In Figure 2 the equilibrium moisture content of spores is plotted against the R.H. at which they were exposed. These results were, as expected, nearly identical to those reported by Nichof, et al.

A comparison of wet and dry side equilibration of Bacillus subtilis var. niger at R.H. of 43% is presented in Figure 3. It is seen that spores equilibrated from the we t side contained appreciably more water than those equilibrated from the dry state. These observations were consistent also with those of other investigations. However, the hys teresis effect coupled with rapid equilibration to different conditions suggested that great care in management of the spore was necessary if a consistent moisture was to be obtained. The dimensions of this problem are demonstrated also in Figure 3. Dry spores were equilibrated at an R.H. of 20% sorbed moisture to the extent of 50 μg H_2O/mg of spores. When, however, in the transfer from the dry state, the spores were exposed to an R.H. of 75% for 30 seconds prior to being placed at an R.H. of 20%; an increase almost 20% in the equilibrium moisture content of the spores was obtained.

The methods used for the determination of spore moisture has been described in the previous quarterly report. We were able to obtain consistently a sensitivity of <0.1 μg of water, a precision of $\pm$ 2 μg H_2O and an accuracy in the neighborhood of 99.9% based on the water content of known amounts of sodium tungstate dehydrate and other hydrated salts. Our experience has been that the greatest single source of error comes from the analytical balance ($\pm$ 0.05 mg).

IV. EFFECT OF SIMULTANEOUS HEATING AND DRYING ON D VALUE

The efficiency of water removed from spores by dry nitrogen in the moisture analyzer suggested the following studies in which the spores were heated and dried simultaneously.

Samples of 1×10^6 spores of Bacillus subtilis var. niger were dried in small thermal death time tubes as previously described. Replicate samples of 3 tubes each were placed into 25 ml serum bottles and equilibrated from the dry side at an R.H. of 43% at 90°F for 24 hours. The bottles were sealed with rubber serum caps while in the equilibration chamber. Under these conditions the spores contained about 10% moisture and the head space above the spores contained about 15 µg H_2O vapor per ml.

The thermal destruction curves were generated in an air oven held at the indicated temperatures ± 0.5°C except at the time when sample changes were made. During these periods the temperature would drop to a maximum of 5°C and about 10 minutes was required to re-establish normal oven temperature. Half of the samples were heated in the sealed serum bottles while the other half were continuously purged with dry N_2 to remove moisture vapor from the head space and the spores. From collateral studies using the moisture balance it was estimated that after 1/2 hr. the moisture content of the spores in the N_2 dried system was less than 0.01% and the moisture in the head space was less than 1 µg H_2O vapor/ml.

The destruction rate of Bacillus subtilis var. niger at 92°C under the above two conditions is shown in Figure 4. It is seen that little or no death occurred in the "moist" control samples. On the other hand a substantial kill was observed in the dry samples.

Similar studies were done at 110, 100, and 80°C and respective D values of <2 hrs, 2 hrs, and 25 hrs were obtained. The controls that were not dried with flowing N_2 showed little inactivation at any of the temperatures studied and meaningful D values could not be calculated.

V. INFLUENCE OF LYOPHILIZATION ON z_D

Previous investigations in this laboratory have demonstrated the effect of various amounts of moisture on the dry heat resistance of Bacillus subtilis var. niger spores. In these studies it was noted that at all temperatures spores in the intermediate moisture range were most heat resistant. However, the only change in z_D occurred when spores were heated in water. Spores in all other moisture levels had z_D values in the dry heat range. In the Sixteenth Quarter the dry side of the spore moisture range was examined to determine if dryness and z_D changed proportionally. Previous attempts at drying spores by lyophilization involved the epoxy embedment system which was suspected of altering environmental conditions during heating. An alternate lyophilization method was studied in this quarter.

Glass strips (1/4" x 7/8") were cut from microscope cover slips, inoculated with 0.01 ml of the aqueous spore suspension (1 x 10^8 spores per glass strip), and dried one hour in a 50°C forced air oven. The strips were placed in tubes which were than constricted one-half inch from the top. These tubes were placed on the manifold of a freeze-dry unit and lyophilized (5 to 15 microns Hg at -40°C) for 12 and 24 hours.

After lyophilization the tubes were sealed under vacuum at the constriction. Duplicate tubes were heated at test temperature, cooled, and aseptically opened. The spores were sampled by grinding the glass strip in a microblender for two minutes. Duplicate plating of each sample was performed as described in the Eleventh Quarterly Report of Progress. All data were corrected for heating and cooling lag.

Lyophilization times of 12 and 24 hours were chosen to determine whether z_D changed with increased drying time. However, 24 hour lyophilization apparently injured the spores and reduced the quality of the data (Table 3). From these rather unreliable D values at 24 hour lyophilization a z_D value of 18.9°C (Figure 5) was calculated.

D values from the 12 hour drying period were more reliable (Table 3) and a z_D of 19.3°C (Figure 6) was determined. z_D's from both drying periods were in the dry heat range and no change was noted with increased dryness. It seems apparent that only two kill mechanisms are involved in heat destruction of spores. Wet heat destruction occurs when spores are water saturated and a dry heat mechanism is involved at all other ranges of moisture.

TABLE 1

Effect of drying and equilibration on the moisture content of *B. subtilis* var. *niger* spores

Procedure used to Prepare Spores	Moisture Content (%) Trial 1	Trial 2
Air dry - 24 hrs. - 40°C	8	-
Air dry - 24 hrs. - 40°C plus N_2 dry - 24 hrs.	2.9	2.6
Same except N_2 dry - 48 hrs.	1.9	1.8
Air dry - 24 hrs. - 40°C, N_2 dry 24 hrs. - 40°C, equilibrated at 41% R.H. 24 hrs.	9.0	9.4
Same as above except equilibrate 48 hrs.	10.0	9.6

TABLE 2

Moisture contained in the can-matrix system and components after equilibration at 41% R.H. at 90°F

System	Equilibration Time (hrs.)	Water (ug) Calculated[a]	Water (ug) Measured
Empty can #1	24	3,100	3,800
Empty can #2	24	3,100	4,100
Empty can #3	24	3,100	3,900
Empty can #4	0.5	3,100	2,900
Can with new matrix	20	1,900	5,500
Can with old matrix	20	1,900	18,400
Can with anodized matrix	20	1,900	55,000
Can with epoxied matrix	20	1,900	12,000
Can with epoxied matrix	20	1,900	18,000
Can with spring clip #1	24	2,700	3,800
#2	24	2,700	4,000
#3	24	2,700	4,200
#4	24	2,700	3,460
#5	24	2,700	3,400
#6	24	2,700	3,660
#7	24	2,700	3,100
#8	45	2,700	4,200
#9	45	2,700	3,300
#10	45	2,700	3,300
#11	45	2,700	3,300
#12	45	2,700	3,500
#13	45	2,700	3,100

(a) Calculation based on theoretical total void volume water content at 41% R.H. at 90°F.

Table 3

Influence of lyophilization on the dry heat resistance of Bacillus subtilis var. niger spores[a]

	12 Hour Lyophilization			24 Hour Lyophilization		
Temperature (°C)	D (min)	95% C.I.[b]	R^2	D (min)	95% C.I.[b]	R^2
105	69.9	66.2 - 74.0	0.97	-	-	-
115	23.0	21.1 - 25.3	0.96	28.5	23.2 - 37.0	0.76
125	6.4	6.1 - 6.8	0.98	8.7	5.4 - 22.8	0.43
135	-	-	-	2.5	1.7 - 5.2	0.65

[a]Inoculum = 1×10^8 spores per sample

[b]C.I. - Confidence Intervals

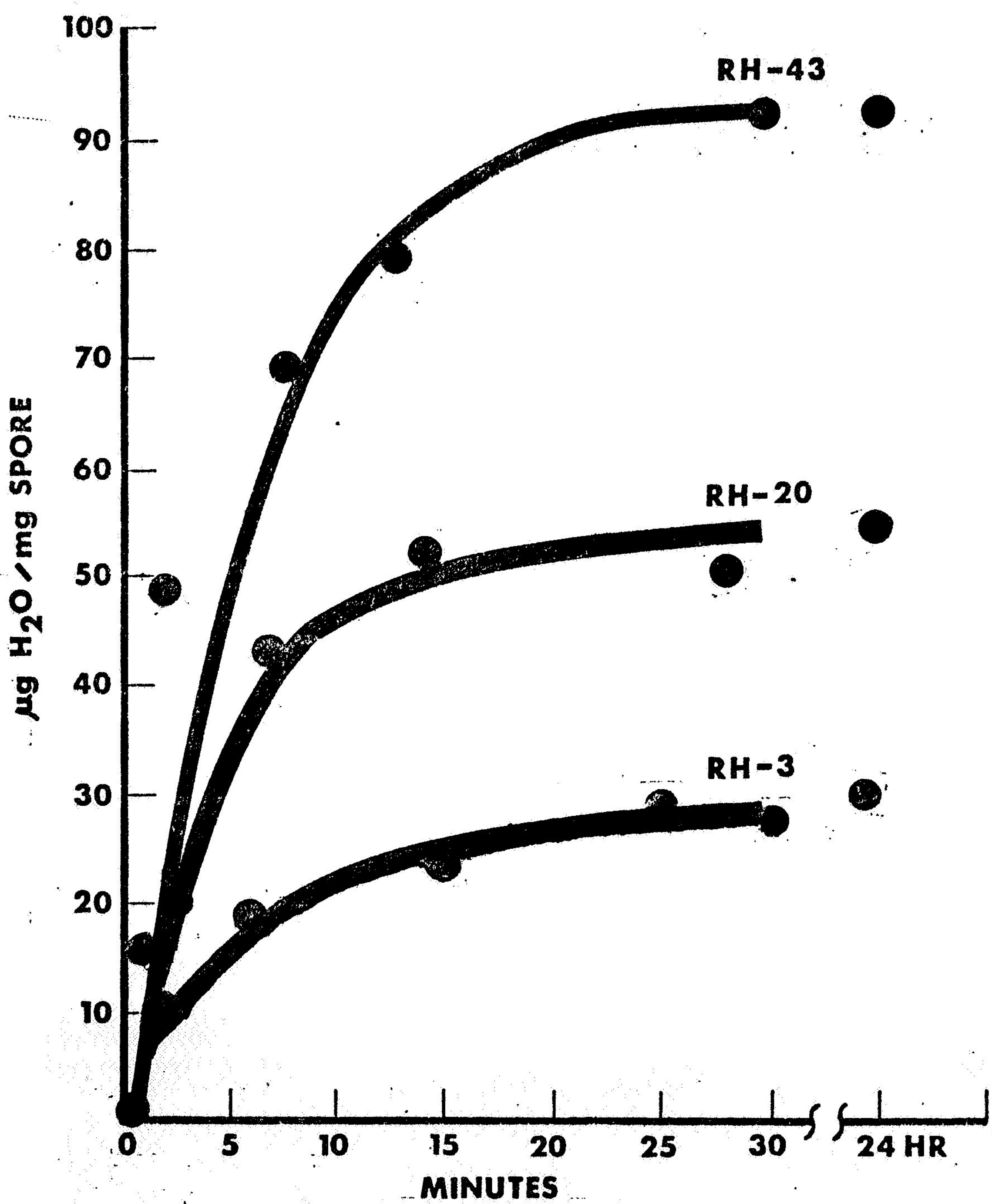

FIGURE 1. Rate of Water Uptake by Bacillus subtilis var. niger

24-26°C

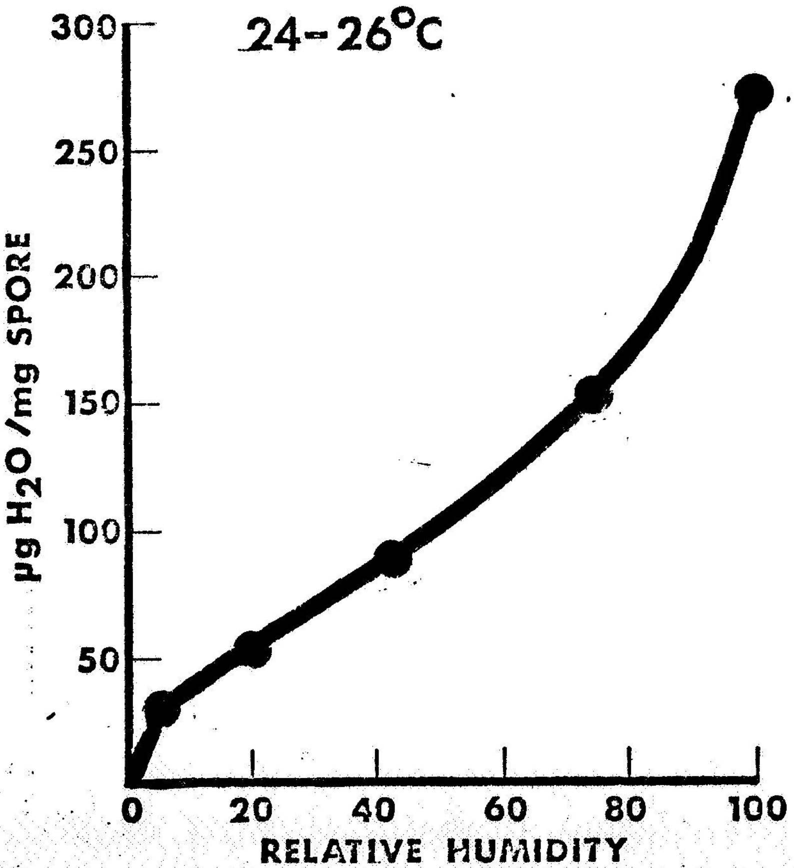

FIGURE 2. Influence of Relative Humidity on the Moisture Content of <u>Bacillus</u> <u>subtilus</u> var. <u>niger</u>

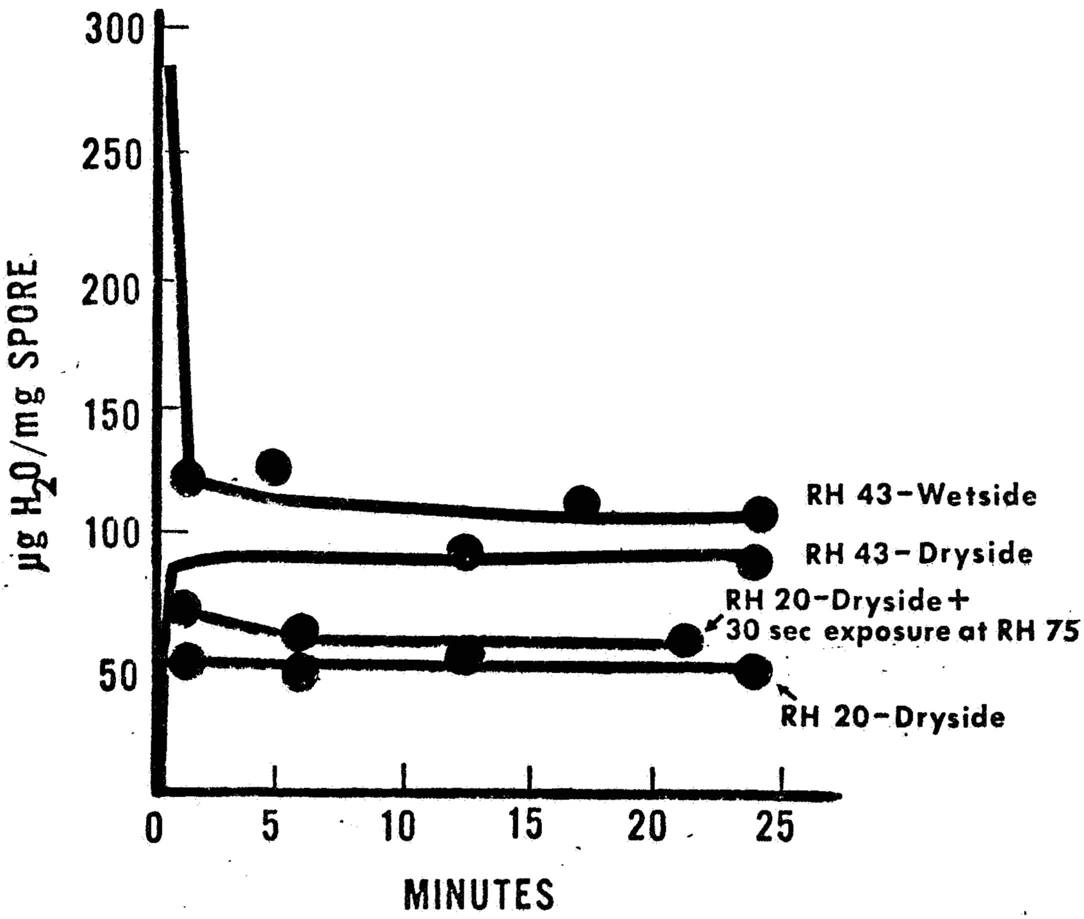

FIGURE 3 **Wet and Dry Side Equilibration of _Bacillus subtilis_ var. _niger_**

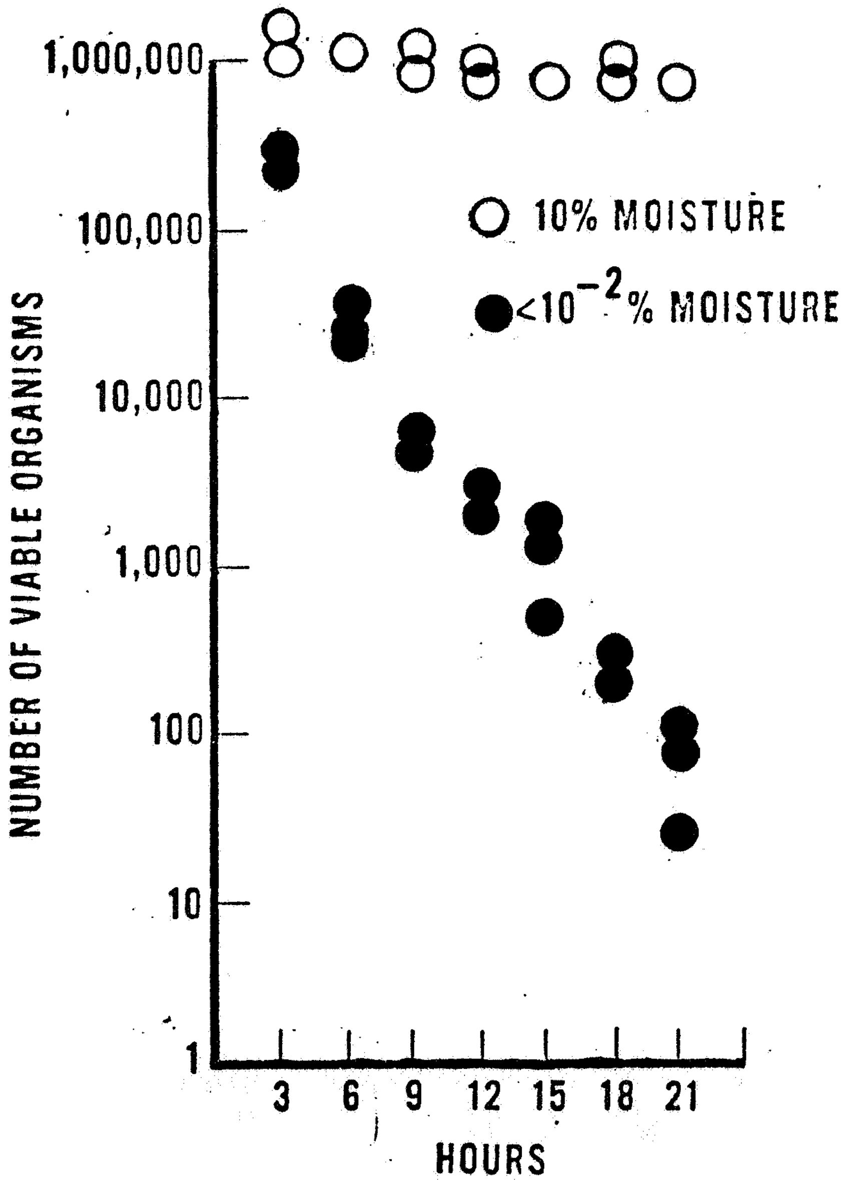

FIGURE 4 **Destruction Rate Curve of _Bacillus subtilis_ var. _niger_ at 92°C**

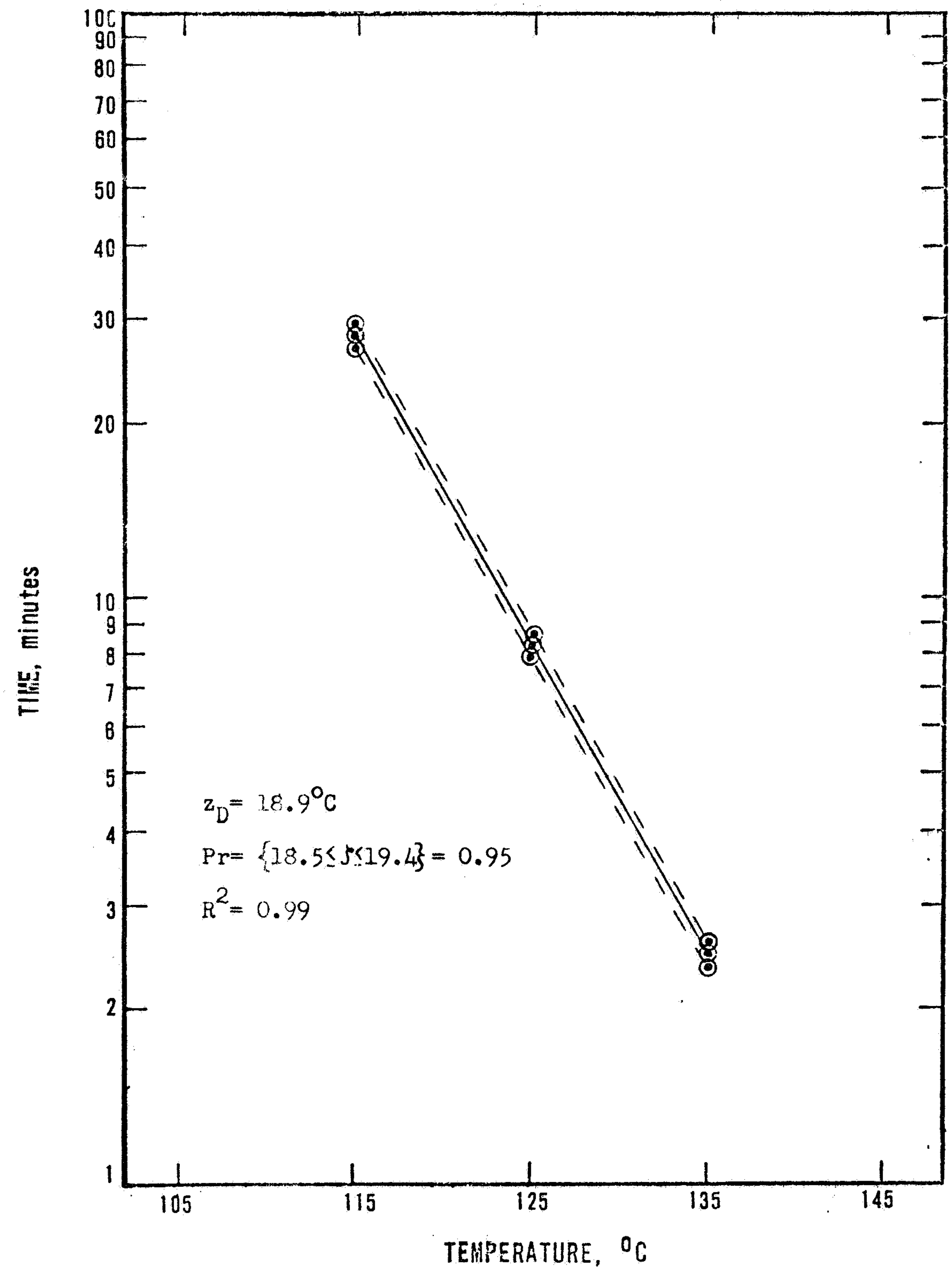

FIGURE 5 Decimal Reduction Time Curve for <u>Bacillus</u> <u>subtilis</u> var. <u>niger</u> Spores Lyophilized Twenty-four Hours

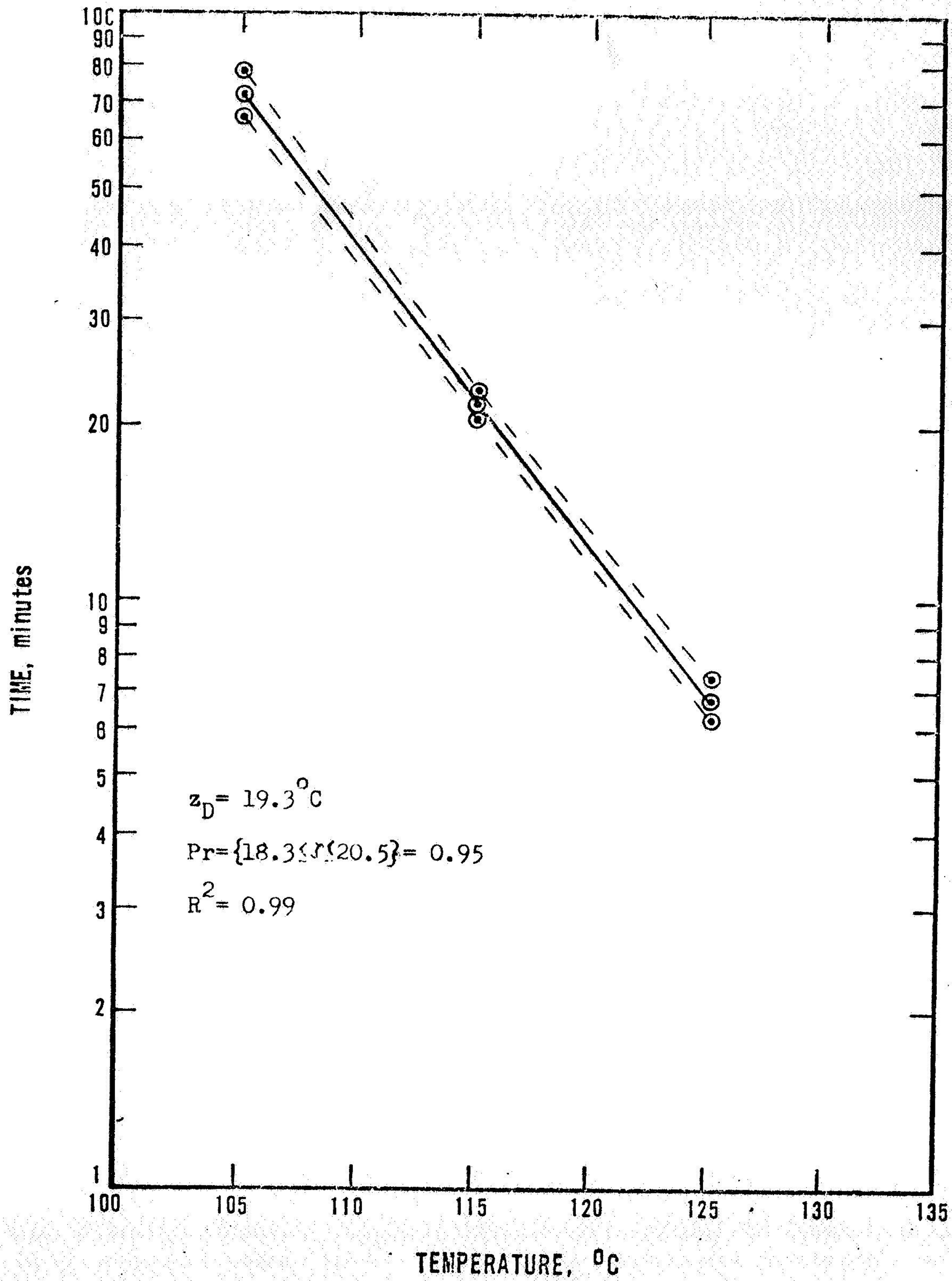

FIGURE 6 Decimal Reduction Time Curve for Bacillus subtilis var. niger Spores Lyophilized Twelve Hours